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Acetylation Phenotype, Carcinogen-Hemoglobin Adducts, and Cigarette Smoking[1]

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ABSTRACT

Levels of 4-aminobiphenyl-hemoglobin adducts in smokers of blonde (flue-cured) and black (air-cured) tobacco have been found to be proportional to bladder cancer risk. In addition, risk of bladder cancer due to exposure to occupational carcinogens is elevated in genetically determined slow acetylators. In this study of normal male volunteers, 4-aminobiphenyl-hemoglobin adducts were found to be related to both the quantity and the type of tobacco smoked, as well as to the acetylator phenotype (independently of smoking habits). The demonstration that both the genetically determined slow acetylator phenotype and tobacco smoking are independently associated with levels of the carcinogen 4-aminobiphenyl in adducted hemoglobin suggests a single mechanism to explain the contribution of genetic susceptibility and environmental exposure in bladder carcinogenesis.

INTRODUCTION

Cigarette smoking is an established cause of urinary bladder cancer, accounting for as much as 50% of the bladder cancer in Western populations (1, 2). Aromatic amines, including ABP[3] and 2-naphthylamine, are found in cigarette smoke and are widely recognized as human and animal carcinogens (3). Two case-control studies have established that smokers of black, air-cured tobacco are at a greater risk of bladder cancer than smokers of blonde, flue-cured tobacco (4, 5). The smoke of black tobacco cigarettes contains more aromatic amines, including ABP, than the smoke of blonde tobacco cigarettes (6). In a previous investigation, we found that the hemoglobin adducts of ABP were approximately 5 times higher in smokers of black tobacco and 3 times higher in smokers of blonde tobacco than in nonsmokers (7). In addition, when adjusted for the same nicotine uptake, smokers of black tobacco excreted twice the amount of mutagenic substances as those smoking blonde tobacco (8). The chemical-biological properties of these mutagens are consistent with their structures being arylamines. The magnitude of increased relative risk of bladder cancer among smokers of the two tobacco types, as compared to nonsmokers, is proportional to the differing concentrations of ABP adducts, consistent with an etiological role of aromatic amines in tobacco-induced bladder cancer.

The metabolism of arylamines is crucial to their ultimate carcinogenic effect. The process of carcinogenesis involves activation to an electrophilic intermediate of the parent arylamine in the liver through *N*-hydroxylation. The *N*-hydroxy metabolite then enters the circulation, reacts covalently with hemoglobin, and is eventually filtered into the bladder lumen, where it reacts with urothelial DNA and initiates tumorigenesis. A competing process of detoxification and elimination proceeds via *N*-acetylation, catalyzed by *N*-acetyltransferase in the liver (9, 10). This noninducible enzyme is under autosomal genetic control; "slow acetylators" are homozygous (rr) for the slow acetylator gene, while "rapid acetylators" are either homozygous or heterozygous (RR, Rr) for the rapid gene (11, 12). A number of studies have indicated that genetically determined slow acetylators are at increased risk of bladder cancer, particularly in subgroups occupationally exposed to aromatic amines (13–20).

MATERIALS AND METHODS

We studied a group of smokers of blonde tobacco, smokers of black tobacco, and nonsmoking subjects to determine whether the level of ABP-hemoglobin adducts in their blood was related to the acetylation phenotype. One hundred healthy male volunteer blood donors between the ages of 45 and 64 years were recruited in Turin, Italy. Informed consent was obtained from subjects, who all underwent phlebotomy for 20 ml of blood, provided a timed urine collection following a dose of caffeine, and responded to a questionnaire on recent and remote smoking, diet, ethnicity, occupation, medication use, and illness. Non-Caucasians were excluded. Of the 100 subjects, 50 were nonsmokers, 31 were smokers of blonde tobacco, 16 were smokers of black tobacco, and 3 smoked pipes. The latter were excluded from the analysis. Blood components and urine were shipped on dry ice to Massachusetts Institute of Technology (Cambridge, MA) for hemoglobin carcinogen adduct assay (S. T. and P. S.) and to the National Center for Toxicological Research (Jefferson, AR) for caffeine metabolite analysis (G. T. and F. K.).

The acetylation phenotype was determined by measuring the urinary excretion of caffeine metabolites following a test dose of the drug. Briefly, subjects received a measured dose of coffee. Four h later, subjects voided and a 1-h timed urine collection was begun. 5-Acetylamino-6-formylamino-3-methyluracil and 1-methylxanthine were determined in an aliquot and their ratio was used to assign the phenotype (rapid acetylator, >0.50; slow acetylator, <0.50), according to a previously published method (21, 22).

The method used to assay ABP adducts is described elsewhere (23). Briefly, hemoglobin derived from washed RBCs undergoes hydrolysis under basic conditions, followed by derivatization to form a pentafluoropropionamide. Capillary gas chromatography with detection by negative ion chemical ionization mass spectroscopy is performed for quantitation of ABP covalently bound as the sulfinic acid amide to cysteine 93β of hemoglobin.

Arithmetic means, SE, correlation coefficients (Pearson), analysis of variance, and linear regression were calculated using the SAS-personal computer software (24).

RESULTS

ABP-hemoglobin adducts showed a bimodal distribution, which is dependent on recent smoking. The mean level of adducts detected was 74 pg/mg HGB in the 97 subjects. Table 1 shows the relationship of adducts to the amount and type of tobacco smoked. ABP adducts were strongly dependent on both the amount and type of tobacco smoked. Fig. 1 shows the relationship of the number of cigarettes smoked in the last 24

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[3] The abbreviation used is: ABP, 4-aminobiphenyl.

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Table 1 *4-Aminobiphenyl hemoglobin adducts by smoking quantity and type of tobacco*

Cigarettes/day	Adducts (pg/g hemoglobin)	
	Black	Blonde
0 (nonsmokers)	28.0 ± 3.0[a]	
1–10	67.0 ± 11.0	61.9 ± 11.1
11–20	151.4 ± 10.4	121.7 ± 17.6
21+	165.8 ± 17.3	123.0 ± 14.3

[a] Mean ± SE.

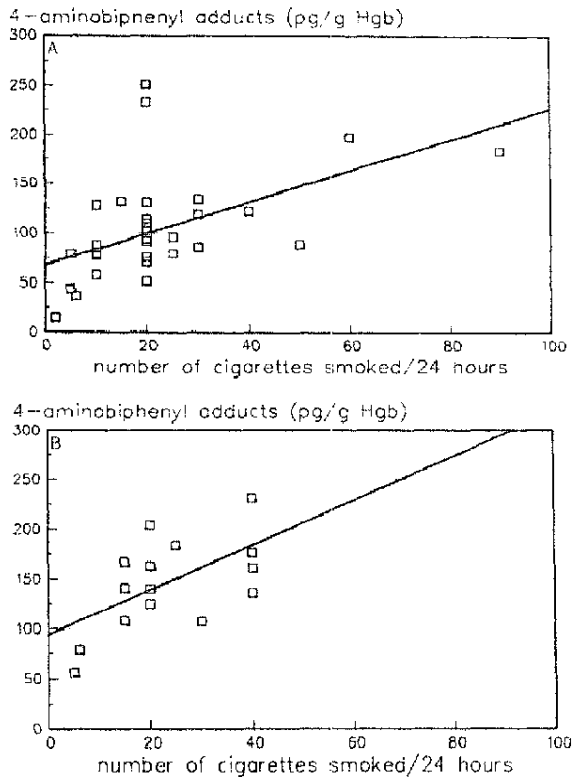

Fig. 1. Relationship of recovered ABP adducts (pg/g hemoglobin) and cigarettes smoked during the prior 24 h in blonde tobacco smokers (*A*) and black tobacco smokers (*B*). A least-squares regression line is shown. For blonde tobacco smokers (*A*): $r^2 = 0.27$, $b = 1.59$, $a = 67.6$, $n = 31$; for black tobacco smokers (*B*): $r^2 = 0.34$, $b = 2.23$, $a = 94.4$, $n = 16$.

h and ABP adducts in blonde tobacco (Fig. 1*A*) and black tobacco (Fig. 1*B*) smokers. When considered separately by linear multivariate methods, both type of tobacco (blonde, $F = 65.1$, $P = 0.0001$; black, $F = 100.4$, $P = 0.0001$) and quantity smoked (cigarettes/day, $F = 111.7$, $P = 0.0001$) demonstrated a strong and independent relation to ABP adducts, while other variables such as age and place of birth (north/south Italy) were unrelated.

There were 63 (65% of the study population) slow acetylators. The 5-acetylamino-6-formylamino-3-methyluracil/1-methylxanthine ratio, used to assign the acetylation phenotype, was unrelated to the number of cigarettes smoked ($r = 0.05$, $P = 0.61$) or to recent caffeine intake, age, race, place of birth, sample shipment group, alcohol, and medication use. The relationship of ABP adducts to the acetylation phenotype was examined by simple and multivariate methods. The mean adduct level in slow acetylators (75.3 pg/g hemoglobin) was higher than in rapid acetylators (64.1 pg/g hemoglobin) when analyzed by univariate methods. When the acetylation phenotype was entered into a linear multivariate model, considering either the study group as a whole or just the smokers, ABP-hemoglobin adducts were found to be significantly related to this genetic trait, after adjustment for both type and quantity of cigarettes smoked (Table 2). Table 3 reports the mean ABP values for acetylation phenotypes and categories of smoking. Higher levels of adducts among slow acetylators were observed in each smoking category, and an association of adduct levels with the type of tobacco was seen within each phenotype. No confounding effect was exerted by the quantity smoked.

DISCUSSION

This study provides the first demonstration that the genetically determined slow acetylator phenotype, which is linked to higher risk for arylamine-induced bladder cancer, is also associated with the detection of the carcinogen 4-aminobiphenyl (a known bladder carcinogen) in adducted hemoglobin derived from cigarette smoke exposure.

Evans (25) has estimated the relative risk of the slow acetylator phenotype for bladder cancer, derived from data from eight earlier epidemiological studies, as being 1.32 (95% confidence interval, 1.02–1.71). The ratio of ABP-hemoglobin adducts detected in slow acetylators compared to rapid acetylators in this study falls within this range for each of the three groups considered (non-, blonde, and black tobacco smokers). Thus, the magnitude of the increase in carcinogen adducts detected in slow acetylators corresponds to the increased risk of bladder cancer associated with the slow acetylator phenotype, as derived from epidemiological studies during the last 15 years.

The relationship between type and quantity of tobacco smoked and detected carcinogen adducts confirms a previous report of this association and is consistent with epidemiological work linking bladder cancer to smoking and type of tobacco (1, 2).

Some "blonde" tobacco cigarettes in Italy are in fact blended with flue-cured, air-cured, and oriental tobaccos, in contrast to blonde cigarettes such as those in Great Britain, which are made entirely from flue-cured tobacco. Future studies on a pure blonde tobacco-smoking group might find an even greater difference in adduct levels, in comparison with black tobacco smokers.

In this biochemical epidemiology study, we have integrated

Table 2 *Multivariate analysis of the relationship of 4-ABP to acetylation phenotype and smoking*

Variable	*F* value	*P* value
Model 1: entire study group, dependent variable = ABP ($n = 97$)		
Acetylation phenotype (slow *vs.* rapid)	9.2	0.003
Type of tobacco (black *vs.* none)	123.2	0.0001
Type of tobacco (blonde *vs.* none)	67.8	0.0001
Age	1.9	0.16
Model 2: smokers only, dependent variable = ABP ($n = 47$)		
Type of tobacco (black *vs.* blonde)	11.6	0.0001
Acetylation phenotype (slow *vs.* rapid)	4.2	0.046
Quantity smoked (cigarette/day)	14.6	0.0004

Table 3 *4-Aminobiphenyl adducts by acetylation phenotype and type of tobacco*

Smoking status	Adducts (pg/g hemoglobin)		Ratio S/R[b]
	Slow[a]	Rapid[a]	
Nonsmokers	31.7 ± 3.8[c]	19.4 ± 4.9	1.6
Blonde tobacco	111.8 ± 13.0	86.4 ± 14.5	1.3
Black tobacco	175.0 ± 11.0	117.5 ± 13.7	1.5

[a] Acetylation phenotype.
[b] Slow/rapid.
[c] Mean ± SE.

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sophisticated laboratory methods into a defined epidemiological framework. A new relationship between macromolecular bound carcinogens, derived from an environmental exposure (arylamines from tobacco), and a genetic risk factor, the acetylation phenotype, has been described.

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